

Cytotoxicity of Nitroaromatic Explosives and their Biodegradation Products in Mice Splenocytes: Implications for their Immunotoxicity

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Nitroaromatic explosives like 2,4,6-trinitrotoluene (TNT) and 2,4,6-trinitrophenyl-*N*-methyl-nitramine (tetryl) comprise an important group of toxic environmental pollutants, whose toxicity is mainly attributed to the flavoenzyme electrontransferase-catalyzed redox cycling of their free radicals (oxidative stress) and DT-diaphorase [NAD(P)H:quinone oxidoreductase, NQO1, EC 1.6.99.2]-catalyzed formation of alkylating nitroso and/or hydroxylamine metabolites. Because of the incomprehensive data on the immunotoxic effects of nitroaromatic explosives, we have studied the structure-cytotoxicity relationships in the action of tetryl, TNT as well as its amino and hydroxylamino metabolites, and related nitroaromatic compounds towards mouse splenocyte cells. The protective effects of desferrioxamine and the antioxidant *N,N'*-diphenyl-*p*-phenylene diamine against the cytotoxicity of TNT and other nitroaromatics showed that the oxidative stress-type cytotoxicity mechanism takes place. In addition, the cytotoxicity of nitroaromatics is also partly prevented by an inhibitor of NQO1, dicumarol. The cytotoxicity of the amino metabolites of TNT is also partly prevented by α -naphthoflavone and isoniazide, which points to the involvement of cytochromes P-450 in their activation. In general the cytotoxicity of nitroaromatics in splenocytes increases with an increase in their single-electron reduction potential, E_1^1 . This points to the prevailing mechanism of the oxidative stress-type cytotoxicity. The obtained structure-activity relationship and the studies of other mammalian cell lines showed that the immunotoxic potential of nitroaromatic explosives may decrease in the order tetryl $\geq$ TNT $\geq$ hydroxylamino metabolites of TNT > amino and diamino metabolites of TNT.

Key words: Nitroaromatic Explosives, Oxidative Stress, Splenocytes, Immunotoxicity

Introduction

Nitroaromatic explosives like 2,4,6-trinitrotoluene (TNT) and 2,4,6-trinitrophenyl-*N*-methyl-nitramine (tetryl) (Fig. 1) are important toxic environmental and workplace pollutants, causing damage to liver, kidney, and spleen, as well as methemoglobinemia in mammalian species (Dilley *et al.*, 1982; Levine *et al.*, 1984, 1990; Reddy *et al.*, 1999). Their toxicity mechanisms involve the oxidative stress caused by the formation of nitroanion

radicals and their redox cycling, which is initiated by flavoenzymes dehydrogenases-electrontransferases, *e.g.* NADPH:cytochrome P-450 reductase (P-450R, EC 1.6.2.4) (Kong *et al.*, 1989; Čėnas *et al.*, 2001), the oxidation of oxyhemoglobin in erythrocytes (Cossum and Rickert, 1987; Marozienė *et al.*, 2001), and the formation of nitroso- and/or hydroxylamino products of two-electron reduction which may modify proteins and DNA, and induce methemoglobin formation (Facchini and Griffiths, 1981; Leung *et al.*, 1995; Šarlauskas *et al.*, 2004).

The effects of nitroaromatic explosives on the mammalian immune system is an important but insufficiently studied problem. The action of nitroaromatic pesticides or industrial pollutants on mammalian species is accompanied by the suppression of the immune response (Dandliker *et al.*, 1980; Burns *et al.*, 1994). Analogously, TNT and tetryl induce splenomegaly in rats and mice (Dilley *et al.*, 1982; Levine *et al.*, 1984, 1990; Reddy *et al.*, 1999) and inhibit splenic macrophage phagocy-

Abbreviations: TNT, 2,4,6-trinitrotoluene; 2-NH₂-DNT, 2-amino-4,6-dinitrotoluene; 4-NH₂-DNT, 4-amino-2,6-dinitrotoluene; 2,4-(NH₂)₂-NT, 2,4-diamino-6-nitrotoluene; 4-NHOH-DNT, 4-hydroxylamino-2,6-dinitrotoluene; cL₅₀, the concentration of compound for 50% cell survival; cI₅₀, the concentration of compound for 50% inhibition; E_1^1 , single-electron reduction potential; P-450R, NADPH:cytochrome P-450 reductase; NQO1, NAD(P)H:quinone oxidoreductase; DPPD, *N,N'*-diphenyl-*p*-phenylene diamine; ROS, reactive oxygen species.

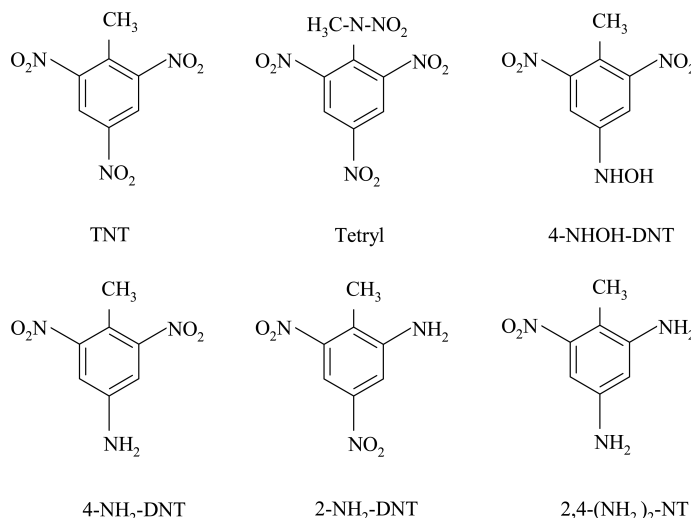


Fig. 1. Chemical structures of explosives and their metabolites studied in this paper.

tosis and production of reactive oxygen species (ROS) (Johnson *et al.*, 2000). TNT also impairs the immune functions of earthworms *Eisenia fetida* (Gong *et al.*, 2007). According to several *in vitro* studies, the products of TNT biodegradation may be less immunotoxic than TNT. The extracts from TNT-contaminated soil inhibited the growth and cytokine production of human peripheral blood mononuclear cells, whereas the biodegradation of TNT partly decreased the inhibition (Beltz *et al.*, 2001). However, these effects were not described quantitatively, and the biodegradation products were not characterized. In a rapid screening assay, 2(4)-amino-4,6(2,6)-dinitrotoluenes [2(4)-NH₂-DNTs] quenched the chemiluminescence of human monocytes less efficiently than TNT; however, another biodegradation product, 2,4-diamino-6-nitrotoluene [2,4-(NH₂)₂-NT] was as efficient as TNT (Bruns-Nagel *et al.*, 1999). This shows that the link between the immunotoxic potential and the structure or the electronic properties of nitroaromatic explosives and related compounds remains insufficiently understood and sometimes controversial, and requires a more thorough evaluation.

Therefore we examined the cytotoxicity of tetryl, TNT as well as its amino and hydroxylamino metabolites (Fig. 1), and related nitroaromatic compounds to mouse splenocyte cells. Our results point to the prevailing oxidative stress-type mechanism of cytotoxicity, and reveal a possible link

between the immunotoxicity and single-electron reduction potential E_1° of nitroaromatic compounds.

Materials and Methods

4- to 8-week-old male and female BALB/c mice [(24 ± 2.0) g] were kept under standard conditions, and were given food and water *ad libitum*. The mice were sacrificed by decapitation, and their spleens were removed according to Stack *et al.* (1999). These experiments were approved by the Lithuanian Veterinary and Food Service (License No. 0171, 2007). Spleens of 3–5 mice for each experiment were used as a source of splenic lymphocytes (splenocytes). Erythrocytes were lysed by 5 min exposure in 3 ml ACK lysis solution (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). After washing the cells twice with RPMI 1640 medium, they were resuspended at the concentration of $1.0 \cdot 10^6$ cells/ml in RPMI 1640 medium with 5% heat-inactivated fetal bovine serum (Sigma), penicillin (100 U/ml), and streptomycin (100 µg/ml), and were used for further experiments. Cell viability was determined after 24 h of incubation of splenocytes with the examined compounds in 96-well cell culture plates (200 µl cell suspension per well) at 37 °C in a humidified atmosphere containing 5% CO₂, according to the Trypan blue exclusion test. The compounds were dissolved in

Table I. The concentrations of explosives, their metabolites, and model nitroaromatic compounds for 50% survival (cL_{50}) of splenocytes determined in the present work, bovine leukemia virus-transformed lamb embryo kidney fibroblasts (line FLK) (Čėnas *et al.*, 2001; Šarlauskas *et al.*, 2004), Chinese hamster ovary KI cells and rat hepatoma H411E cells (Honeycutt *et al.*, 1996), Chinese hamster lung V-79 cells and human lymphoblast TK-6 cells (Lachance *et al.*, 1999), their concentrations for 50% inhibition of chemiluminescence of monocytes (cI_{50}) (Bruns-Nagel *et al.*, 1999), and their single-electron reduction potentials ($E_1^\ddagger$).

Compound	cL_{50} [μM]					cI_{50} [μM]		$E_1^\ddagger$ [V] ^a
	Splenocytes		FLK	KI	H411E	V-79	TK-6	
Tetryl (1)	6.0 ± 1.5	1.5	1.5 ± 0.3					-0.156
2,4,6-Trinitrotoluene (2)	10 ± 2.0	25	25 ± 5.0					-0.253
p-Dinitrobenzene (3)	2.5 ± 0.4	8.0	8.0 ± 2.0	106	17.6	197 ± 36	22.0 ± 5.0	-0.257
o-Dinitrobenzene (4)	60 ± 15	30	30 ± 5.0					-0.287
p-Nitrobenzaldehyde (5)	40 ± 6.0	25	25 ± 12					-0.325
m-Dinitrobenzene (6)	100 ± 20	90	90 ± 20					-0.348
p-Nitroacetophenone (7)	60 ± 8.0	166	166 ± 36					-0.355
2-NH ₂ -DNT (8)	750 ± 50	440	440 ± 35	>1270	91.3	222 ± 76	168 ± 14	-0.417
4-NHOH-DNT (9)	112 ± 10	100	100 ± 20	18.8	28.2			-0.429
4-NH ₂ -DNT (10)	500 ± 80	316	316 ± 20	>1270	335	>328	248 ± 51	-0.449
p-Nitrobenzyl alcohol (11)	650 ± 90	2000	2000 ± 400					-0.475
Nitrobenzene (12)	1000 ± 200	4370	4370 ± 1370					-0.485
2,4-(NH ₂) ₂ -NT (13)	270 ± 30	350	350 ± 40	>1500	>1500	>600	>600	-0.502

^a From Wardman (1988); Riefler and Smets (2000); Šarlauskas *et al.* (2006).

DMSO, whose final content in the medium, 0.6%, did not affect the splenocyte viability.

For the determination of the enzyme activity, cells were sonicated on ice in four cycles of 10 s each. The homogenate was centrifuged at 16000 × g for 45 min. The protein concentration was determined according to the method of Bradford. The activity of P-450R was determined spectrophotometrically using a Hitachi-557 spectrophotometer in 0.1 M K-phosphate (pH 7.0) with 1 mM EDTA at 25 °C, according to the reduction rate of 50 μM cytochrome *c* ($\Delta\epsilon_{550} = 20 \text{ mm}^{-1} \text{ cm}^{-1}$) by 100 μM NADPH. The activity of DT-diaphorase was determined by the reduction of 50 μM dichlorophenolindophenol ($\Delta\epsilon_{600} = 21 \text{ mm}^{-1} \text{ cm}^{-1}$) with 100 μM NADPH, as the difference between the rates in the absence and presence of 20 μM dicumarol. In this assay, Tween 20 (0.01%) and bovine serum albumin (0.25 mg/ml) were added as activators.

Tetryl, TNT and its amino and hydroxylamino metabolites were synthesized by Dr. Jonas Šarlauskas (Institute of Biochemistry, Vilnius, Lithuania) as described previously (Čėnas *et al.*, 2001; Šarlauskas *et al.*, 2004). The purity of the compounds was checked by their melting point and elemental analysis, IR and NMR spectra. All the other compounds were obtained from Sigma or Aldrich and used as received. The statistical analysis was performed using Statistica (version 4.3, StatSoft, 1993).

Results and Discussion

We studied the cytotoxicity of tetryl, TNT and its amino- and hydroxylamino metabolites (Fig. 1), and model nitroaromatic compounds in mouse splenocytes. The concentrations of compounds for 50% cell survival (cL_{50}) are given in Table I. For comparison, previously determined cL_{50} values of TNT and its metabolites in several other mammalian cell lines (Honeycutt *et al.*, 1996; Lachance *et al.*, 1999; Čėnas *et al.*, 2001; Šarlauskas *et al.*, 2004) and their concentrations for 50% inhibition of chemiluminescence (cI_{50}) of human monocytes (Bruns-Nagel *et al.*, 1999) are also included. The mammalian cell cytotoxicity of nitroaromatic compounds frequently increases with an increase in their single-electron reduction potential ($E_1^\ddagger$) with coefficient $\Delta \log cL_{50}/\Delta E_1^\ddagger \sim -10 \text{ V}^{-1}$ (O'Brien *et al.*, 1990; Čėnas *et al.*, 2001, and references cited therein). In these cases the oxidative stress-type

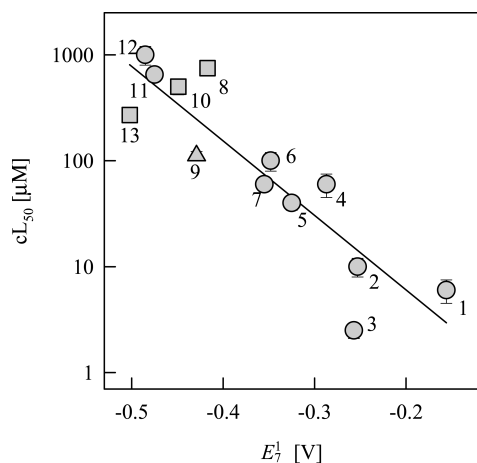


Fig. 2. Dependence of cytotoxicity of nitroaromatic compounds in splenocytes on their single-electron reduction potential, $E_{1/2}^1$, according to equation (1). The numbers of compounds are taken from Table I.

cytotoxicity mechanism may prevail, because the single-electron reduction of nitroaromatics by flavoenzymes dehydrogenases-electrontransferases, *e.g.* NADPH:cytochrome P-450 reductase (P-450R), which may initiate their redox cycling, is characterized by a coefficient $\Delta \log$ (rate constant)/ $\Delta E_{1/2}^1 \sim 10 \text{ V}^{-1}$ (Orna and Mason, 1989; Čėnas *et al.*, 2001). We found that the activity of P-450R in splenocytes is equal to $(11 \pm 1.0) \text{ nmol cytochrome } c \text{ reduced}/(\text{mg protein} \cdot \text{min})$. The data of

Table I show that the cytotoxicity of nitroaromatic compounds in splenocytes increases with an increase in their $E_{1/2}^1$ value (Fig. 2), and is described by the regression:

$$\log cL_{50} [\mu\text{M}] = -(0.627 \pm 0.401) - (7.406 \pm 1.060) E_{1/2}^1 [\text{V}] \quad (1)$$

$(r^2 = 0.800, F(1,11) = 44.140).$

As in our previous studies (Čėnas *et al.*, 2001; Šarlauskas *et al.*, 2004), the toxicity of TNT and tetryl in splenocytes was partly decreased by the antioxidant *N,N'*-diphenyl-*p*-phenylene diamine (DPPD) (Ollinger and Brunmark, 1991) and the iron-chelating agent desferrioxamine, the latter preventing the Fenton reaction (Fig. 3A).

Another important mechanism of activation of nitroaromatic compounds is their two(four)-electron reduction by DT-diaphorase [NAD(P)H:quinone oxidoreductase, NQO1, EC 1.6.99.2] to hydroxylamines (Misevičienė *et al.*, 2006, and references cited therein). The activity of NQO1 in splenocytes is equal to $(4.0 \pm 1.0) \text{ nmol dichlorophenolindophenol reduced}/(\text{mg protein} \cdot \text{min})$. The toxicity of tetryl, TNT, and several dinitrobenzenes in splenocytes was partly decreased by an inhibitor of NQO1, dicumarol (Fig. 3B). In contrast, dicumarol did not affect the toxicity of 2-NH₂-DNT, 4-NH₂-DNT, and 2,4-(NH₂)₂-NT (data not shown). It is related most probably to the low

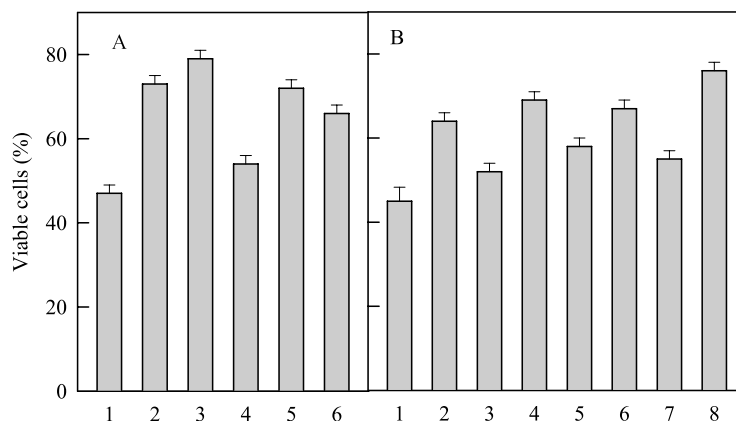


Fig. 3. (A) The protecting effects of DPPD ($3 \mu\text{M}$) and desferrioxamine ($300 \mu\text{M}$) in the cytotoxicity of $10 \mu\text{M}$ TNT and $6.0 \mu\text{M}$ tetryl in splenocytes. 1, TNT; 2, TNT + DPPD; 3, TNT + desferrioxamine; 4, tetryl; 5, tetryl + DPPD; 6, tetryl + desferrioxamine; $n = 3$; $p < 0.01$ for 2, 3 against 1, and for 5, 6 against 4. (B) The protecting effect of dicumarol ($30 \mu\text{M}$) against the toxicity of nitroaromatic compounds. 1, $10 \mu\text{M}$ TNT; 2, $10 \mu\text{M}$ TNT + dicumarol; 3, $6.0 \mu\text{M}$ tetryl; 4, $6.0 \mu\text{M}$ tetryl + dicumarol; 5, $100 \mu\text{M}$ *m*-dinitrobenzene; 6, $100 \mu\text{M}$ *m*-dinitrobenzene + dicumarol; 7, $2.5 \mu\text{M}$ *p*-dinitrobenzene; 8, $2.5 \mu\text{M}$ *p*-dinitrobenzene + dicumarol; $n = 3$; $p < 0.02$ for 1, 3, 5, 7 against 2, 4, 6, 8. Cell viability in control experiments, $(98 \pm 2)\%$.

activity of the above compounds as the substrates for NQO1 (Šarlauskas *et al.*, 2004).

Previously, we have found that the FLK cell cytotoxicity of amino and hydroxylamino metabolites of TNT was higher than one may expect from their reduction potential (Šarlauskas *et al.*, 2004). In splenocytes, the cL_{50} values of NH_2 -DNTs do not show significant deviation from the regression, although the toxicity of 4-hydroxylamino-2,6-dinitrotoluene (4-NHOH-DNT) and 2,4-(NH_2)₂-NT seems to be slightly enhanced (Fig. 2). Apart from their redox cycling, NHOH-DNTs may exert an additional mode of cytotoxicity, the alkylation of DNA. In turn, the enhanced cytotoxicity of amino metabolites of TNT may be attributed to the formation of corresponding hydroxylamines under the action of cytochromes P-450 (Kim *et al.*, 2004). The exclusion of cL_{50} values of NH_2 -DNTs, 2,4-(NH_2)₂-NT, and 4-NHOH-DNT (compounds **8–10**, **13**, Table I) from the regression improves the correlation

$$\log cL_{50} [\mu M] = -(0.791 \pm 0.421) - (7.570 \pm 1.233) E_1^1 [V] \quad (2)$$

$$(r^2 = 0.844, F(1,7) = 37.719).$$

Indeed, the inhibitor of cytochromes P-450 1A1/2 and 1B1, α -naphthoflavone (Shimada *et al.*, 1998), and the inhibitor of cytochrome P-450 2E1, isoniazide, partly decreased the toxicity of 2- NH_2 -DNT and 2,4-(NH_2)₂-NT, whereas the toxicity of TNT decreased less significantly (Fig. 4). The expression of cytochrome P-450 1B1 in mouse splenocytes has previously been demonstrated (Miyata *et al.*, 2001). In control experiments, dicumarol, α -naphthoflavone and isoniazide did not decrease the cytotoxicity of 200–400 μM H_2O_2 [$cL_{50} = (420 \pm 50) \mu M$, data not shown].

Conclusions

The data of this work enable us to make several conclusions on the immunotoxic potential of nitroaromatic explosives and their metabolites *in vitro*, which may be possibly important under the conditions *in vivo* as well:

(i) The splenocyte cytotoxicity of nitroaromatic explosives (tetryl, TNT) and their metabolites increases with an increase in their E_1^1 values (Table I, Fig. 2). This may also imply that tetryl may be more immunotoxic than TNT (Table I). Taken together with the antioxidant protection (Fig. 3A), these data point to the prevailing mechanism of

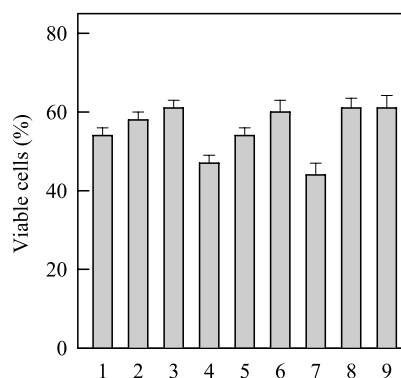


Fig. 4. The protecting effects of α -naphthoflavone (5 μM) and isoniazide (1 mM) in the cytotoxicity of 10 μM TNT, 700 μM 2- NH_2 -DNT, and 250 μM 2,4-(NH_2)₂-NT. 1, TNT; 2, TNT + α -naphthoflavone; 3, TNT + isoniazide; 4, 2- NH_2 -DNT; 5, 2- NH_2 -DNT + α -naphthoflavone; 6, 2- NH_2 -DNT + isoniazide; 7, 2,4-(NH_2)₂-NT; 8, 2,4-(NH_2)₂-NT + α -naphthoflavone; 9, 2,4-(NH_2)₂-NT + isoniazide; $n = 3$; $p < 0.05$ for 1 against 2, 3; $p < 0.02$ for 4 and 7 against 5, 6, and 8, 9.

the oxidative stress-type cytotoxicity, which was previously evidenced in FLK cells (Čėnas *et al.*, 2001) and hepatocytes (O'Brien *et al.*, 1990). Although the inhibitor of NQO1, dicumarol, also exerts protective effects in splenocytes (Fig. 3B), the reactions of NQO1 may be a minor factor in their cytotoxicity, because the reactivity of nitroaromatics in NQO1-catalyzed reactions does not follow the well-expressed dependence on their E_1^1 (Misevičienė *et al.*, 2006, and references cited therein).

(ii) In splenocytes, like in other cell lines, the products of degradation of TNT, NH_2 -DNTs are less toxic than the parent compounds (Table I), although the degree of the cytotoxicity decrease is highly variable. This probably reflects the superposition of two opposite factors, a decrease in the ROS-formation rate by NH_2 -DNTs due to a decrease in their E_1^1 (Šarlauskas *et al.*, 2004) and their activation by cytochromes P-450 (Fig. 4), which was first demonstrated in this work. Interestingly, 2,4-(NH_2)₂-NT was found to be much less toxic than TNT in splenocytes, lymphoblasts and in other mammalian cell lines, although it quenched the monocyte chemiluminescence as efficiently as TNT (Table I). However, the biochemical mechanisms of the latter phenomenon were not disclosed (Bruns-Nagel *et al.*, 1999). Irrespective of the reasons for this discrepancy, we suppose that a longer incubation time used in the cytotoxic-

ity assays may more closely resemble the natural conditions, *i.e.*, the chronic intoxication by TNT and its metabolites, than the data of 30 min chemiluminescence experiments.

(iii) 4-NHOH-DNT is less toxic than TNT in splenocytes and FLK cells, whereas it is similarly or even more toxic than TNT in the other two cell lines investigated (Table I). In our opinion, this discrepancy may be attributed to the additional factor of the cytotoxicity of 4-NHOH-DNT, the direct reactions with DNA (Kim *et al.*, 2004, and references cited therein), whose cytotoxic consequences may depend on the cell type. Nevertheless, the results of our study support the suggestion that the products of biodegradation of TNT are less immunotoxic than TNT (Beltz *et al.*, 2001). We

suggest that the net immunotoxicity of the studied explosives and their metabolites to mammalian species in general increases with an increase in their electron-accepting potency, and follows the order tetryl $\geq$ TNT $\geq$ hydroxylamino metabolites of TNT $>$ amino and diamino metabolites of TNT.

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